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Identification of Novel Genes and Pathways Affecting *Salmonella* Type III Secretion System 1 Using a Contact-Dependent Hemolysis Assay▽

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We screened 5,700 *Salmonella enterica* serovar Typhimurium mutants for defects in type III secretion system 1 (T3SS-1)-mediated contact-dependent hemolysis to identify novel genes and pathways affecting the activity of T3SS-1. Our data suggest that previously unrecognized factors such as type I fimbriae may modulate the expression, activity, or deployment of this key virulence factor.

Type III secretion systems (T3SSs) are found in many gram-negative bacteria and are used to secrete and translocate a range of bacterial proteins known as effectors directly into the host cell (reviewed in reference 6). *Salmonella* possesses two T3SSs which are used at distinct stages of the infection process. T3SS-1, encoded within *Salmonella* pathogenicity island 1 (SPI-1), is required for the initial invasion of host cells and the induction of enteropathogenic responses (reviewed in 14, 22), while T3SS-2, encoded within SPI-2, is used in the later stages of infection, promoting survival and replication of *Salmonella* within host cells (reviewed in reference 1).

Lysis of erythrocytes in a manner dependent upon T3SSs has been observed in a number of bacteria including *Shigella*, enteropathogenic *Escherichia coli*, *Yersinia*, *Bordetella* and *Salmonella* (8, 10, 12, 15, 23). In *Salmonella*, hemolysis is dependent upon the ability of the T3SS-1 translocator proteins SipB, SipC, and SipD to form a pore in the erythrocyte membrane, causing hemoglobin leakage (15). In vitro, contact-dependent hemolysis can be monitored by incubating *Salmonella* strains with sheep red blood cells (SRBC) and measuring the absorbance of the culture supernatant to detect the presence of released hemoglobin (15). In this work we used a high-throughput contact-dependent hemolysis assay to screen a *Salmonella enterica* serovar Typhimurium mutant bank with the aim of identifying novel genes or pathways involved in regulation of *Salmonella* T3SS-1.

Screening serovar Typhimurium mutants for contact-dependent hemolysis. An *S. enterica* serovar Typhimurium 4/74 nalidixic acid-resistant (Nal^r) signature-tagged transposon insertion mutant (STM) bank of 5,700 independent mutants (17) was screened. Bacterial strains in 96-well plates grown overnight in Luria Bertani (LB) medium at 21°C were subcultured and incubated at 37°C for 90 min to induce expression of T3SS-1. Bacteria were recovered by centrifugation, resuspended in fresh LB medium, and assessed for their ability to lyse SRBC essentially as described previously (15). Briefly, defibrinated sheep blood (TCS Biosciences Ltd., Buckingham, United Kingdom) was centrifuged at 5,700 × *g* for 5 min at 4°C to pellet RBC. The RBC were washed four times in phosphate-buffered saline (PBS) and then resuspended in an equal volume of PBS to give a 50% (vol/vol) SRBC-PBS suspension. Fifty microliters of bacterial culture was mixed with 50 μl of SRBC-PBS suspension in a 96-well plate and centrifuged at 1,279 × *g* for 10 min to aid contact between bacteria and SRBC. Following a 4-h incubation at 37°C, cells were resuspended by the addition of 150 μl of PBS and centrifuged at 1,279 × *g* for 10 min. A total of 100 μl of supernatant from each well was transferred to a flat-bottomed 96-well plate, and the absorbance was recorded at 550 nm to detect the presence of released hemoglobin.

From this initial screen of 5,700 serovar Typhimurium 4/74 mutants, 447 showed decreased hemolytic activity (approximately 60% or less activity compared to the plate average), of which 174 were classed as negative since they were unable to induce lysis of SRBC. The value of 60% was chosen following assessment of variance in the assay. Following the initial screen, any mutants showing reduced hemolysis were screened three more times in the hemolysis assay to confirm the phenotype. To allow for variation between biological replicates in the additional screens, each plate contained a positive (serovar Typhimurium 4/74 wild type) and negative control. Absorbance values were adjusted to remove background hemolysis using a negative control of RBC without bacteria. Results were then expressed as hemolytic activity as a percentage of wild-type bacteria (positive control), where the wild type was classed as inducing 100% hemolysis. Mutants with hemolytic activity of less than 60% were classed as showing decreased hemolysis compared to wild-type bacteria, and mutants with a value of 10% or less were classed as negative (unable to induce hemolysis). The optical density at 550 nm (OD_{550}) of the bacterial cultures was measured prior to incubation with the SRBC in the hemolysis assay, and some mutants with reduced growth (40 to 60% of wild-type) were retested in the hemolysis assay with bacterial numbers adjusted so that they were similar to the wild-type strain. Mutants with substantially impaired growth rates were rejected for further analysis. Pilot studies indicated that the extent of hemolysis was growth phase dependent, with bacteria in early or mid-logarithmic phase being

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TABLE 1. Mutations outside of SPI-1 resulted in impaired T3SS-1-mediated contact-dependent hemolysis by *S. enterica* serovar Typhimurium

Unique mutant identifier	Gene (synonym)	Function	Hemolytic activity (% of wild type [±SD]) in:	
			STM	P22 transductant
M7f4	*hns*	DNA binding protein, global regulator	13.0 ± 4.7[b]	
M17c10	*stpA*	HNS-like global regulator	39.7 ± 26.6	
M28f10, M45f10, M49d10[c]	*sirA* (*uvrY*)	Response regulator controlling virulence gene expression	5.2 ± 6.7	20.02 ± 13.8
M60f1	*rfaL*	LPS core biosynthesis; O-antigen ligase	23.9 ± 4.8[b]	
M7a4	*rfaG*	LPS core biosynthesis; glucosyltransferase I	47.9 ± 5.9[b]	
M37f12	*rfaI*	LPS core biosynthesis; (glucosyl)lipopolysaccharide-alpha-1,3-D-galactosyltransferase	3.6 ± 5.3[b]	
M5b4	*rfbU*	LPS O-antigen biosynthesis	34.7 ± 6.9	
M31f1	*rfbI*	LPS O-antigen biosynthesis	46.9 ± 7.8	
M33d10, M38d10	*rfbF*	LPS O-antigen biosynthesis	30.1 ± 16.2[b]	
M31g3	*cpsB* (*rfbM*, *manC*)	Mannose-1-phosphate guanyltransferase, involved in colanic acid biosynthesis	41.4 ± 24.4	48.34 ± 10.7
M14h4	*spr* (*yeiV*)	Putative lipoprotein, suppresses thermosensitivity of *prc* mutants at low osmolarity	56.3 ± 17.6	
M16f3	*fimW*	Regulator of type I fimbriae expression	42.9 ± 4.9	25.3 ± 11.5[d]
M12d5, M14g3, M14h5, M13e9, M37e9	*fadD*	Long chain fatty acid acyl-CoA synthetase	16.8 ± 22.6	51.9 ± 0.9[d]
M56c5	*ackA*	Acetate kinase A	48.0 ± 14.1[b]	27.05 ± 4.3
M49g1	*aroD*	3-Dehydroquinate dehydratase	45.2 ± 13.9[b]	61.71 ± 19.8
M54e12	*deaD*	ATP-independent RNA helicase	67.2 ± 22.4[b]	49.86 ± 15.2
M34c4	*uvrB*	Part of ATP-dependent DNA excision repair enzyme complex	41.6 ± 20.8	
M41a5,[c] M47a5, M54a5	*agaR*	Transcriptional repressor of *aga* operon	53.0 ± 14.3	87.47 ± 19.3
M46g10	*envC* (*yibP*)	Peptidoglycan hydrolase; required for cell division	39.1 ± 6.6	
M53h8	*yqjB (ecfM)*	Unknown	55.3 ± 12.8	
M44d6, M14h10	*yfgA*/*yfgB*[a]	Unknown	69.8 ± 15.5[b]	
M50d1	*ycgL*/*minC*[a]	Unknown (*ycgL*); cell division inhibitor (*minC*)	61.0 ± 27.1	

[a] Transposon insertion between the two genes.
[b] Results obtained from additional assay with bacteria corrected to the same OD_{550}.
[c] STM mutant used for P22 transduction.
[d] Defined deletion mutant was used rather than a P22 transductant.

less hemolytic than those in late exponential phase. Though hemolytic activity was sensitive to growth phase, it was not affected by the starting bacterial density as diluting cultures through 80, 60, 40, and 20% of the starting OD_{550} did not impair hemolytic activity. Thus, if equivalent numbers of bacteria enter the assay and if mutants with impaired growth are rejected, one may anticipate that mutants with altered hemolytic activity carry mutations that directly affect the expression, function, or deployment of T3SS-1.

Identification of mutants with reduced T3SS-1-mediated hemolytic activity. For the remainder of this study we focused on mutants that we had classified as reduced or negative in the hemolysis screen.

Since a large number of mutants exhibiting impaired hemolytic activity were predicted to have defects in SPI-1, the chromosomal DNA region encoding T3SS-1, a PCR screen was performed to identify and eliminate such mutants. Four primers spanning the T3SS-1 apparatus-encoding region of SPI-1 (T3SS-P01, 5′-TGC CTGCTATTCAGGAAACA-3′; T3SS-P02, 5′-CAGGATGGG AGGCTATTCAA-3′; T3SS-P03, 5′-CACCCATGATGGCGTA TAGA-3′; and T3SS-P04, 5′-TTTTCGCCAGGACGATATTC-3′) were used in PCRs with the transposon-specific primers P6 (5′-CCTAGGCGGCCAGATCTG-3′) and P10 (5′-TCCTCTA GAGTCGACCTG-3′). Genomic DNA from each mutant was prepared according to standard procedures and used as a template in the PCRs. Any mutant that generated a product was discarded as the transposon insertion would be in an SPI-1 gene. This method eliminated approximately 50% of the mutants. The remaining mutants were then identified by subcloning and sequencing to map transposon insertion sites as previously described (17) with the exception that sequencing was carried out in-house using Beckman CEQ8000 sequencers.

Twenty-three genes outside of SPI-1 were identified and are listed in Table 1. Some genes were previously known to affect T3SS-1 activity, for example, *sirA* encoding a global response regulator (11). This provided further evidence that the hemolysis assay was a suitable method for identifying genes and pathways affecting T3SS-1. Genes not previously known to affect *Salmonella* T3SS-1 were involved in a number of different biochemical pathways including lipopolysaccharide (LPS) biosynthesis, fimbriae expression, and DNA repair. Multiple independent mutations in some systems were identified; for example, six separate genes in the LPS biosynthetic pathway were identified, confirming the importance of this pathway in T3SS-1-mediated hemolysis and markedly reducing the likelihood that the observed phenotypes are due to second-site defects. Further, the assay reproducibly detected the involvement of specific genes; for example, *fadD* mutants were isolated five times, and *sirA* mutants were isolated three times. The serovar Typhimurium LT2 genome was analyzed to identify which mutated genes were in operons and therefore potentially sensitive to polar effects. Of those genes identified as being located in an operon and therefore having potential polar effects, the majority were in LPS genes. The involvement

of LPS per se in regulation of T3SS-1 activity should be regarded with caution as it is required for membrane integrity and proper insertion and folding of membrane proteins. Indeed, some lipid A mutations have already been shown to impair T3SS-1 function (24).

To confirm that the phenotype of reduced T3SS-1-mediated hemolysis was due to mutation of the gene identified by transposon insertion sequencing and not to secondary defects, P22 transduction of a subset of the mutants into the archived serovar Typhimurium 4/74 parent strain was performed. Mutation of the desired gene in the resulting transductants was confirmed by PCR with gene-specific primers. The P22 transductants were retested three times in the hemolysis assay and displayed a similar phenotype as the original STM mutant (Table 1), confirming that the effect on T3SS-1-dependent hemolysis was most likely due to disruption of the gene identified by sequencing of the transposon insertion.

For further validation of the screen, *fadD* was selected as this gene had previously been identified in a screen of mutants with reduced expression of the T3SS-1 transcriptional regulator *hilA* (13). *fadD* encodes the long-chain fatty acyl-coenzyme A (CoA) synthetase, which is associated with the inner bacterial membrane and catalyzes the esterification of long-chain fatty acids into fatty acyl-CoA molecules (reviewed in reference 4). The fatty acyl-CoA molecules can be incorporated into phospholipids for membranes or can be further degraded via the beta-oxidation pathway. A defined *fadD* deletion mutant was constructed using overlapping PCR followed by allelic exchange with the pDM4 suicide vector (16). The first 300 and last 325 nucleotides of *fadD* were amplified by PCR using the primer pair FadD_5′_xho (5′-CTCCTCGAGTTGAAGAAGGT TTGGCTT-3′) and FadD_int2 (5′-CAGAATCATATCTTTCT TAATCATGCCAGCGCGTAAAATACC-3′) and the pair FadD_3′_xba (5′-CTCTCTAGATCAGGCTTTATTGTCTACT TT-3′) and FadD_int3 (5′-TTACGCGCTGGCATGATTAAGA AAGATATGATTCTGGTTTCT-3′; restriction sites are underlined). PCR products were used in an overlapping PCR using FadD_5′_xho′ and FadD_3′_xba primers, and the resulting product was subcloned into the pCR2.1-TOPO vector (Invitrogen) and then cloned into pDM4 using XhoI and XbaI restriction sites. The resulting plasmid pDM4-*fadD* was introduced into serovar Typhimurium 4/74 Nal^r (17) by conjugation from *E. coli* S17.1λ*pir*, and merodiploids were selected on LB agar containing chloramphenicol (25 μg/ml) and nalidixic acid (20 μg/ml). Double recombinants were selected by growing merodiploids to late log phase in LB medium, plating on LB agar lacking NaCl and containing 6% (wt/vol) sucrose and nalidixic acid, and incubation at 30°C. *S. enterica* serovar Typhimurium 4/74 Nal^r Δ$fadD_{300–1360}$ mutants were verified by colony PCR to lack nucleotides 300 to 1360 of the *fadD* gene, leaving behind the first 300 and last 325 nucleotides of the gene.

The hemolysis assay was repeated with the defined *fadD* mutant, and as with the original STM mutants, the defined mutant induced less lysis (51.9% ± 0.9%) of SRBC than serovar Typhimurium 4/74 Nal^r wild-type (Table 1), further validating results of the screen of transposon mutants for those with impaired T3SS-1-dependent hemolytic activity.

Characterization of the effect of *fimW* mutation on T3SS-1-dependent hemolysis. Having identified genes which affect the activity of *Salmonella* T3SS-1, we sought to characterize one of these mutants. *fimW* was selected because this gene had not previously been linked to type III secretion, but its function is well characterized. FimW is a negative regulator of type I fimbriae in serovar Typhimurium (21), which, in turn, contribute to adherence of bacteria to eukaryotic cells and in some cases facilitate invasion (3, 5, 7). Type I fimbriae are composed of FimA subunits which form a fimbrial shaft tipped by the FimH adhesin, which mediates binding to mannose moieties on eukaryotic cells (5, 9).

To confirm the effect of mutation of *fimW* on T3SS-1-mediated contact-dependent hemolysis, a whole-gene deletion mutant was constructed using a similar method as for the *fadD* mutant with the primer pair *fimW*_xho_5′ (5′-CTCCTCGAGCT TGCACCTAATCATTTAAC-3′) and *fimW*_mut_3′ (5′-CCTTGTAAAAAGTTAAGTGAGTTTACATTGCGCCAGA AAAGACGATATTC-3′) and the pair *fimW*_xba_3′ (5′-CTCTC TAGAGGAAGTTGCGTCAGGACCTC-3′) and *fimW*_mut_5′ (5′-ATATCGTCTTTTCTGGCGCAATGTAAACTCACT TAACTTTTTACAAGGC-3′; restriction sites are underlined). The resulting mutant, serovar Typhimurium 4/74 Nal^r Δ*fimW*, contained just the start and stop codons of the *fimW* gene. For use as a comparison in some assays a *fimA* deletion mutant lacking the fimbrial subunit (serovar Typhimurium 4/74 Nal^r Δ*fimA*) was also created using the same method with the primer pair *fimA*_xho_5′ (5′-CTCCTCGAGATGCGACATT TTATACAAAA-3′) and *fimA*_mut_3′ (5′-GCCGTTCCCTG ACGGGATTACATGGATTTCCCTTGAATTACACAC-3′) and the pair *fimA*_xba_3′ (5′-CTCTCTAGAACGAAAATGG AACGCTGACG-3′) and *fimA*_mut_5′ (5′-TGTAATTCAAG GGAAATCCATGTAATCCCGTCAGGGAACGGCAGG-3′).

The expected phenotypes of the mutants were confirmed by blotting and mannose-sensitive yeast agglutination. For the dot blot, bacterial strains were cultured statically in 10 ml of LB medium containing nalidixic acid (20 μg/ml) at 37°C for 48 h. Whole-cell lysates were prepared by centrifuging 10 ml of culture, washing the bacterial pellet in PBS, and resuspending it in 50 μl of PBS. The lysates were normalized to equivalent bacterial numbers, serial dilutions were made, and samples were heated at 95 to 100°C for 8 min. Four-microliter spots were applied to Hybond ECL membrane (GE Healthcare) in two applications of 2 μl. The membrane was blocked in 3% nonfat dried milk in PBS, and immunoblotting was performed using anti-FimA-glutathione *S*-transferase antiserum (1:100), a kind gift from Andreas Bäumler, and goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:10,000; Sigma). Type I fimbriae were detected in the serovar Typhimurium 4/74 Nal^r wild-type sample at a dilution of up to 1:200 (Fig. 1). In contrast, in the serovar Typhimurium 4/74 Nal^r Δ*fimW* mutant, type I fimbriae were still detected at the highest dilution of 1:500, indicating that this strain produces an elevated level of FimA and, presumably therefore, more type I fimbriae than the wild-type strain. FimA was not detected in the serovar Typhimurium 4/74 Nal^r Δ*fimA* strain.

As the dot blot assay used whole-cell lysates and could not distinguish surface-expressed type I fimbriae, a yeast cell slide agglutination assay was performed based on an adaptation of a method by Roe et al. (19). Briefly, bacterial strains were cultured overnight statically at 21°C and then diluted 1:2 into 10 ml of fresh LB medium and cultured statically for 90 min at 37°C. These culture conditions mimicked those of the hemo-

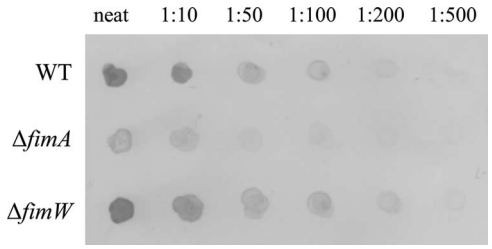

FIG. 1. Analysis of the expression of FimA by serovar Typhimurium wild-type and *fimW*-defined deletion mutant. Bacteria were grown statically at 37°C for 48 h. Whole-cell lysates of the cultures were prepared, serially diluted, and heated at 95 to 100°C for 8 min. Protein dot blots were performed using anti-FimA-glutathione *S*-transferase antiserum, an alkaline phosphatase-conjugated anti-rabbit secondary antibody, and the BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) detection method. WT, wild type.

lysis assay, and in addition to providing data on surface expression levels of type I fimbriae, the assay provided data that could be correlated to type I fimbrial expression patterns in the hemolysis assay. Bacteria were pelleted by centrifugation and resuspended in PBS, with the OD_{600} of the cultures adjusted to 1.75. Agglutination was assayed on glass slides mixing 15 μl of bacteria with an equal volume of 10 mg/ml *Saccharomyces cerevisiae* cell suspension (Sigma). Slides were incubated at 4°C and monitored periodically for agglutination. Mannose inhibition of agglutination was confirmed using 3% (wt/vol) D-mannose in the yeast suspension. Serovar Typhimurium 4/74 Nal^r Δ*fimW* displayed strong yeast cell agglutination within 10 min, whereas serovar Typhimurium 4/74 Nal^r wild-type bacteria displayed agglutination only after a 20-min incubation. The serovar Typhimurium 4/74 Nal^r Δ*fimA* mutant, as expected, did not agglutinate yeast cells. A negative control of enterohemorrhagic *E. coli* O157:H7 (TUV930), which does not express type I fimbriae (19), did not cause yeast cell agglutination. Addition of 3% (wt/vol) D-mannose abolished agglutination in all cases, indicating that yeast cell agglutination in the serovar Typhimurium 4/74 Nal^r Δ*fimW* mutant and parent strain was due to surface expression of type I fimbriae.

In the hemolysis assay the defined *fimW* mutant induced less lysis (25.3% ± 11.5%) of SRBC than serovar Typhimurium 4/74 Nal^r wild type (Table 1). Based on these data, we hypothesized that a possible mechanism of action for the role of type I fimbriae in T3SS-1 activity is a spatial interference effect, whereby the increased amount of type I fimbriae on the surface of the *fimW* mutant prevents the T3SS-1 from efficiently contacting eukaryotic cells. Consistent with this hypothesis, removal of type I fimbriae via mutation of *fimA* resulted in increased hemolysis (113.2% ± 23.0%) compared to the wild-type strain.

The *fadD* and *fimW* mutants behaved differently from wild-type bacteria in another T3SS-1-dependent assay. A second read-out of T3SS-1 function, the gentamicin protection assay, was carried out to measure bacterial invasion of cultured epithelial cells since invasion is a major function of *Salmonella* T3SS-1.

Bacterial strains were cultured statically overnight at 25°C in 10 ml of LB medium containing appropriate antibiotics and then diluted 1:2 in 10 ml of fresh LB medium and cultured statically for 90 min at 37°C. Confluent monolayers of HeLa cells in 24-well plates were washed in PBS and incubated for 30 min at 37°C in 5% CO_2 in fresh Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum. Approximately 5×10^5 bacteria were added to each well of HeLa cells (multiplicity of infection of 2.5), and plates were centrifuged to facilitate contact of the bacteria with the HeLa cells. Following a 1-h infection at 37°C in 5% CO_2, cells were washed three times in PBS and fresh Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and 150 μg/ml gentamicin was added. Cells were incubated for 1 h more and then were washed three times in PBS and lysed by the addition of 100 μl of 1% Triton X-100 in PBS for 5 min. PBS (900 μl) was added to each well, and bacteria were plated onto L-agar plates containing nalidixic acid (20 μg/ml) for enumeration of invaded bacteria. Each bacterial strain was assayed in triplicate in the invasion assay.

S. enterica serovar Typhimurium 4/74 Nal^r Δ$fadD_{300–1360}$ showed an 80% decrease in invasion compared to serovar Typhimurium 4/74 Nal^r wild-type (Fig. 2a), and a control of serovar Typhimurium 4/74 harboring a transposon insertion in a gene encoding a structural component of T3SS-1, *prgH* (*prgH*::miniTn*5*Km2) (17), was unable to invade HeLa cells,

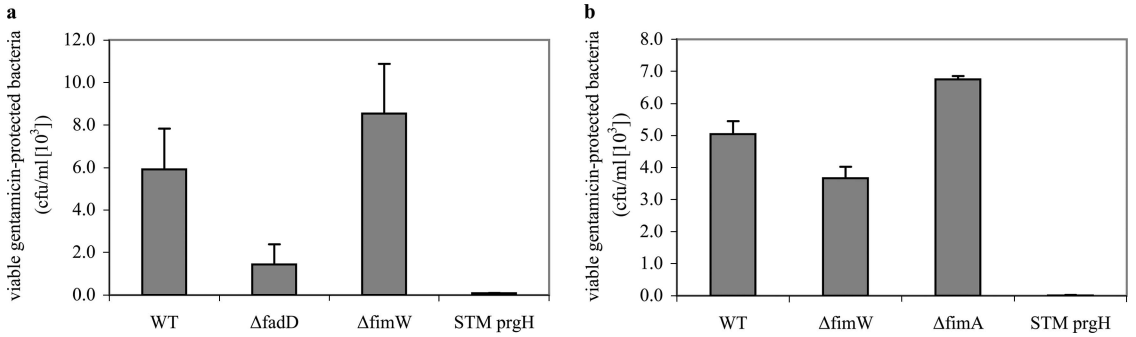

FIG. 2. Mutations in type I fimbrial genes affect invasion in cultured epithelial cells. Bacteria were grown statically overnight at 21°C, subcultured at 1:2, and temperature shifted to 37°C for 90 min to induce expression of T3SS-1. Confluent monolayers of HeLa cells in 24-well plates were infected with 5×10^5 CFU/well. At 1 h postinfection cells were washed with PBS, and fresh medium containing 150 μg/ml gentamicin was added for a further hour. Cells were lysed with 1% Triton X-100, and bacteria were enumerated by plating on L-agar. Invasion assays were performed in the absence (a) and presence (b) of D-mannose. Values represent a typical experiment performed with triplicate samples; error bars indicate standard error of the mean. WT, wild type.

indicating that the assay was dependent on a functional T3SS-1. Under these conditions, serovar Typhimurium 4/74 Nal^r Δ*fimW* was more invasive than the wild-type strain (Fig. 2a), contrasting with data from the hemolysis assay. However, when the invasion assay was repeated in the presence of 0.2 M D-mannose to block secondary effects of the FimH adhesin, serovar Typhimurium 4/74 Nal^r Δ*fimW* showed a 25% decrease in invasion compared to the wild-type strain (Fig. 2b). The FimH adhesin of type I fimbriae binds mannosylated glycoproteins on or secreted by eukaryotic cells, facilitating adhesion of bacteria to eukaryotic cells; this observation suggests that in the initial invasion assay (Fig. 2a), results were biased by increased type I fimbriae on the *fimW* mutant strain, thereby resulting in increased adherence to the HeLa cells. This is supported by reports that *Salmonella* type I fimbriae mediate adhesion in vitro to a variety of cell types (3, 7, 20) and contribute to colonization of pigs in vivo (2).

The differences in the ability of the *fadD* and *fimW* mutants to induce T3SS-1-mediated contact-dependent hemolysis may be partially due to differences in T3SS-1 secretion. Protein secretion by *Salmonella* T3SS-1 can be induced in vitro by a temperature shift of the bacterial culture from 25°C to 37°C, resulting in the secretion of large quantities of T3SS-1 effector proteins into the culture supernatant (26). We used a modification of this method to mirror the culture conditions used in the hemolysis assay. Briefly, bacterial strains were cultured statically overnight in 10 ml of LB medium at 25°C, diluted 1:2 into 10 ml of fresh LB medium, and cultured statically for 90 min at 37°C. Ten milliliters of culture was pelleted by centrifugation (10,000 × *g* for 10 min) and resuspended in 7.5 ml of fresh LB medium to remove the background of secreted proteins. Five milliliters was added to 5 ml of PBS and incubated statically for 4 h at 37°C. At the end of the incubation period, bacteria were pelleted by centrifugation, and the supernatant was passed through a 0.45-μm-pore-size low-protein-binding filter. Secreted proteins were collected by incubating the filtered supernatant with 30 μl of StrataClean resin (Stratagene). The resin was washed in PBS and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue. Western blotting of secreted proteins was performed using Hybond-ECl membrane, blocked in 3% (wt/vol) nonfat dried milk and incubated with anti-SipC (clone F569-cc6c) or anti-SipB (clone F575-AB4) monoclonal antibodies (generated in-house) at 1 μg/ml (diluted in 1% [wt/vol] bovine serum albumin in Tris-buffered saline), followed by donkey anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) at a concentration of 1:10,000. Blots were developed using chemiluminescence (ECL kit, GE Healthcare).

Secreted protein profiles from both serovar Typhimurium 4/74 Nal^r Δ*fimW* and serovar Typhimurium 4/74 Nal^r Δ$fadD_{300–1360}$ showed reduced amounts of T3SS-1-secreted proteins (Fig. 3a), with the *fadD* mutant showing the least amount of secreted proteins. Western blotting confirmed that both the *fadD* mutant and the *fimW* mutant secreted less SipB and SipC T3SS-1 translocator proteins than the wild-type strain (Fig. 3b). Samples of the bacterial cultures were taken at 0 and 4 h postinoculation for viable counts. Bacterial numbers were similar for all cultures at each time point; thus, the mutations did not affect bacterial growth (data not shown).

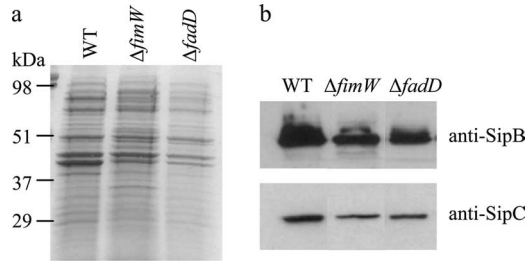

FIG. 3. The *fimW* and *fadD* mutants secrete smaller amounts of T3SS-1-secreted proteins than wild-type bacteria. Bacteria were grown statically overnight at 21°C, subcultured 1:2 into 20 ml, and cultured statically at 37°C for 90 min. Ten milliliters of culture was pelleted by centrifugation and resuspended in 7.5 ml of fresh LB medium, and 5 ml was added to 5 ml of PBS and incubated statically for 4 h at 37°C. Secreted proteins were isolated from culture supernatants and subjected to SDS-PAGE followed by Coomassie staining (a) or Western blotting with anti-SipC and anti-SipB antibodies (b). WT, wild type.

Concluding comments. A screen of 5,700 serovar Typhimurium 4/74 random transposon mutants identified a number of novel mutations that have impaired T3SS-1 activity, as judged by a decrease in T3SS-1-mediated contact-dependent hemolysis compared to the parent strain. We used a defined *fadD* mutant to validate the screen, and our findings from the hemolysis assay and other T3SS-1-dependent assays correlate with data by Lucas and colleagues, which identified *fadD* in a Tn*5* mutagenesis screen for mutants affecting expression of the T3SS-1 transcriptional activator *hilA* (14). Interestingly, Lucas et al. found that *fadD* affected *hilA* expression independently of the fatty acid regulator *fadR* (13). One possible hypothesis is that this may represent a novel mechanism that *Salmonella* has evolved to utilize the conventional fatty acid pathway to sense the environment and regulate virulence gene expression accordingly.

For *fimW*, which had not previously been implicated in T3SS-1 activity, we confirmed the impaired T3SS-1 activity observed in the hemolysis assay using additional T3SS-1-dependent assays. Analysis of T3SS-1-secreted proteins from both the *fadD* and *fimW* mutants revealed that the decreased T3SS-1 activity correlated with a decrease in the levels of secreted proteins compared to wild-type bacteria. In particular, SipB and SipC were reduced in both mutants, and these proteins are required for translocation of other T3SS-1-secreted proteins into eukaryotic cells and are also responsible for the T3SS-1-dependent hemolysis by forming a pore in the erythrocyte membrane (15).

The mechanism of action that explains how disrupting a gene involved in regulation of type I fimbriae expression leads to a decrease in secretion of T3SS-1 proteins is unclear. We initially hypothesized that the impaired ability of the *fimW* mutant to induce hemolysis was due to a spatial interference effect, whereby the increased amount of fimbriae on the bacterial surface of a *fimW* mutant prevents T3SS-1 from efficiently contacting and lysing erythrocytes. However, analysis of T3SS-1-secreted proteins indicated that this is not the sole explanation as the amounts of T3SS-1-secreted proteins collected from the culture supernatant of the *fimW* mutant were less than the amount from the wild-type strain. Thus, there seems to be a more complex, multifactorial explanation, and at

this stage we are unable to entirely rule out some role for spatial interference or effects on membrane integrity. Indeed, it is likely that other genes we identified in the hemolysis screen that are involved in bacterial surface structures such as LPS may have a spatial interference explanation for their lack of hemolysis, either impeding the ability of T3SS-1 to contact erythrocytes or affecting assembly of T3SS-1 in the bacterial membrane. The effect of LPS mutations on type III secretion has been observed in *Shigella*, where a mutant defective in LPS O-antigen glucosylation showed reduced epithelial cell invasion in vitro compared to wild-type bacteria (25). This is because glucosylation of LPS causes a conformational change in the O antigen, shortening the distance it extends from the *Shigella* surface and thereby optimizing access of the T3SS to the epithelial cells (25). In *Yersinia* increasing the length of the adhesin YadA reduced Yop effector translocation via the T3SS (18), providing further evidence of how bacterial surface structures impact on T3SS function.

Other genes identified in our screen may have more complex, and as yet unknown, explanations for their inability to induce T3SS-1-mediated contact-dependent hemolysis, and characterizing these mutants could potentially identify new targets for therapeutics.

This work has shown that the *Salmonella* T3SS-1-dependent hemolysis assay developed by Miki and colleagues (15) is a useful method for high-throughput screening of a large number of mutants for T3SS-1 activity. The method has numerous advantages including speed, reliability, and sensitivity. It should be capable of being readily adapted to other bacteria demonstrating T3SS-dependent hemolytic activity to identify novel genes and pathways affecting type III secretion in these organisms.

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